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Studies on Steroids in Urine of the Male Newborn

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Key Words. Urine of the newborn · Steroids · Chromatography · Hydrolysis

Abstract. Four different methods of isolation and purification were utilized to study steroids in urine of male newborns which was collected during the first 5 days of life. These methods included celite column, ion exchange column and thin-layer chromatography, solvolysis and enzyme hydrolysis with β-glucuronidase and aryl sulfatase. Procedural losses were evaluated by using radioactive internal standards. Final quantitation of each steroid was achieved by comparison of its chromatographic and quantitative behavior with the respective standard steroids on various gas-liquid chromatography systems, either as parent compound or as trimethylsilyl ether derivative. The following steroids were found in the amounts indicated: progesterone, 2.1 μg/l (pool I), 4.6 μg/l (pool III); pregnanediol, 625.0 μg/l (pool IIa), 605.0 μg/l (pool IIb glucuronide), 25.4 μg/l (pool IIb sulfate), 4.2 μg/l (pool IIb free), 729.0 μg/l (pool III); 16α-hydroxyprogesterone, 713.0 μg/l (pool III); 16α-hydroxypregnenolone, 14,000.0 μg/l (pool III); 16α-hydroxydehydroepiandrosterone, 2,350.0 μg/l (pool III); 16-dehydroprogesterone, 155.0 μg/l (pool I), 21.2 μg/l (pool IIb glucuronide), 97.5 μg/l (pool IIb sulfate), 5.3 μg/l (pool III); 16-dehydropregnenolone, 382.0 μg/l (pool I), 1,380 μg/l (pool IIb glucuronide), 172.0 μg/l (pool IIb sulfate), 174.0 μg/l (pool III); 16-dehydropregnanolone, 8.3 μg/l (pool I), 239.0 μg/l (pool IIb sulfate). Pregnenolone, pregnanolone, 17α-hydroxyprogesterone and 17α-hydroxypregnenolone could not be detected. The results support the concept that the steroid patterns of urine of the newborn and amniotic fluid are very similar and that the amniotic fluid steroid content is mainly dependent on fetal urinary steroid excretion. The data on Δ^{16}-C_{21}-steroids are discussed.

Introduction

Studies on amniotic fluid have demonstrated a high concentration of 16-hydroxylated steroids compared with the respective precursors (1, 2). In cord blood, however, these precursors are present in rather large quantities (3–5). This has been confirmed by our own investigations (6, 7). Newborn urine data

published so far indicate that, as in amniotic fluid, 16-hydroxylated steroids are found to be present in high concentrations and the precursors in only minute quantities (8–10). Based upon these results, we have postulated that the amniotic fluid steroid pattern is mainly dependent on fetal urinary excretion (2, 6). Since the data published on newborn urine steroid concentrations show some discrepancies, we have isolated, identified and quantitated a number of steroids in newborn urine using different methodological approaches. Among these steroids, three Δ^{16}-C_{21}-steroids will be described.

Materials and Methods

Urine from 50 normal male newborns was collected overnight in plastic bags (Braun, Melsungen) for the first 5 days of life. There was no contamination of urine by feces. The urine was stored frozen at −20 °C until processed. All solvents used were of reagent grade and obtained from Merck (Darmstadt), except for isooctane (Fluka).

Trivial names and abbreviations. Progesterone (P) = 4-pregnen-3,20-dione; pregnanediol (P-diol) = 5β-pregnan-3α,20α-diol; pregnenolone (Pe) = 3β-hydroxy-5-pregnen-20-one; pregnanolone (Pa) = 3α-hydroxy-5β-pregnan-20-one; 16-dehydroprogesterone (16-DP) = 4,16-pregnandien-3,20-dione; 16-dehydropregnenolone (16-DPe) = 3β-hydroxy-5,16-pregnenedien-20-one; 16-dehydropregnanolone (16-DPa) = 3β-hydroxy-16,(5β)-pregnen-20-one; 17α-hydroxyprogesterone (17OH-P) = 17α-hydroxy-4-pregnen-3,20-dione; 17α-hydroxypregnenolone (17OH-Pe) = 3β,17α-dihydroxy-5-pregnen-20-one; 16α-hydroxyprogesterone (16OH-P) = 16α-hydroxy-4-pregnen-3,20-one; 16α-hydroxypregnenolone (16OH-Pe) = 3β,16α-dihydroxy-5-pregnen-20-one; 16α-hydroxydehydroepiandrosterone (16OH-D) = 3β,16α-dihydroxy-5-androsten-17-one; dehydroepiandrosterone (D) = 3β-hydroxy-5-androsten-17-one.

Reference steroids. The purity of the following reference steroids was evaluated by gas-liquid chromatography (GLC) analysis, if possible, as trimethylsilyl ether (TMSi) derivatives: P[i] (Ikapharm); Pa (Mann Research Laboratory); Pe, P-diol, 17OH-P, 17OH-Pe (Ikapharm); 16OH-P, 16OH-Pe, 16OH-D, 16-DP, 16-DPe, 16-DPa (Steraloids).

Radioactive steroids. In order to correct for procedural losses and to trace and identify the various steroids throughout the extensive isolation and purification procedures, the purity of all the radioactive steroids was evaluated by celite column or thin-layer chromatography prior to use as internal standards. The following labelled steroids were used: Progesterone-7α-^{3}H, spec. act. 9.6 C/m*M* (Radiochemical Centre, Amersham); pregnanolone-1,2-^{3}H, spec. act. 32.7 C/m*M* (New England Nuclear Corp.); pregnenolone-7α-^{3}H, spec. act. 6.9 C/m*M* (Radiochemical Centre, Amersham); pregnanediol-1,2-^{3}H, spec. act. 50.3 C/m*M* (New England Nuclear Corp.); 17α-hydroxyprogesterone-7α-^{3}H, spec. act. 14.8 C/m*M* (Radiochemical Centre, Amersham); 17α-hydroxypregnenolone-7α-^{3}H, spec. act. 19.6 C/m*M* (Radiochemical Centre, Amersham); 16α-hydroxyprogesterone-4-^{14}C, spec. act. 52.8 mC/m*M* (Dr. *Siiteri*), 16α-hydroxypregnenolone-4-^{14}C, spec. act. 52.4 mC/m*M* (Dr. *Siiteri*).

Determination of radioactivity. The radioactivity was measured in Packard liquid scintillation spectrometers model 3380, 2002 and 314 EX. Counting was carried out in polyethylene vials (NEN Plastic LSC Vial NeF-938). Liquid scintillator was prepared by mixing 40 ml Packard Permafluor 25 × with 1 liter of reagent grade toluene. Using radioactive internal standards, procedural losses were corrected by counting an aliquot prior to

Table I. Solvent systems for chromatography

Chromatography system	Composition	Ratio	Number of developments
Celite column			
C-1	ethylene glycol:isooctane: ethylene acetate (gradient)		
Thin-layer (Silica gel G)			
T-1	ethyl acetate	–	1
T-2	ethyl acetate:benzene	9:1	2
T-3	ethyl acetate:cyclohexane	6:4	1
T-4	benzene:ethyl acetate	2:1	1
T-5	benzene:ethyl acetate	9:1	1
T-6	chloroform	–	2
T-7	chloroform	–	3
T-8	chloroform:acetone	8:2	1
T-9	chloroform:acetone	8:2	2
T-10	chloroform:acetone	9:1	2
T-11	chloroform:ethanol	9:1	2
T-12	chloroform:ethyl acetate	13:1	1
T-13	chloroform:ethyl acetate	13:1	2
T-14	chloroform:benzene	8:2	1
T-15	chloroform:benzene	8:2	3
T-16	chloroform:dioxane	94:6	1
T-17	cyclohexane:ethyl acetate	1:1	1
T-18	cyclohexane:ethyl acetate	6:4	1
T-19	methylene chloride:ether	96:4	1
T-20	methylene chloride:ether	96:4	2
T-21	methylene chloride:ether	9:1	1
T-22	methylene chloride:acetone	4:1	1
T-23	methylene chloride:dioxane	19:1	1

quantitation. To localize the radioactivity coming off the columns, $^1/_{30}$ of every second fraction was counted. On thin-layer plates radioactivity was located with the thin-layer scanner Berthold LB 2723.

Chromatography. Celite column chromatography was carried out as described by *Silteri* (11) and detailed recently by *Schindler* (2). Amberlite column chromatography was performed according to *Bradlow* (12) and *Shackleton et al.* (13). Plates for thin-layer chromatography were prepared as previously described (14). The solvent systems used for chromatography are summarized in table I.

Gas-liquid chromatography. A Packard gas chromatograph 7401/564 and a Hewlett Packard gas chromatograph 5750 were used for GLC. The columns were coiled with a length of 180 cm and an internal diameter of 4 mm. The support material consisted of Gas-Chrom Q and was coated with 2–3 % XE-60, QF-1, OV-1, OV-3, OV-7 or OV-17. The

Ratio	Number of developments
–	1
9:1	2
6:4	1
2:1	1
9:1	1
–	2
–	3
8:2	1
8:2	2
9:1	2
9:1	2
13:1	1
13:1	2
8:2	1
8:2	3
94:6	1
1:1	1
6:4	1
96:4	1
96:4	2
9:1	1
4:1	1
19:1	1

umns, $^1/_{50}$ of every second located with the thin-layer

rried out as described by olumn chromatography was (13). Plates for thin-layer he solvent systems used for

h 7401/564 and a Hewlett ns were coiled with a length material consisted of Gas-)V-3, OV-7 or OV-17. The

carrier gas consisted of nitrogen. The operating temperatures were as follows: column 210–230 °C, injector 240–260 °C, dedector 240–250 °C. Quantitation was carried out according to *Adlercreutz and Luukkainen* (15). TMSi derivative formation was performed as described before (2). For final identification gas chromatograph – mass spectrometry was carried out as described elsewhere (16).

Experimental and Results

Three separate pools of urine from newborns were used in this investigation. The second pool was subdivided into two parts. An outline of the procedures applied is indicated in table II.

It is important to note that the strong acid and alkaline conditions used on pools I and II have been completely avoided when processing pool III.

Table II. Outline of the investigation

Pool I 2,850 ml	Pool II 1,650 ml		Pool III 2,600 ml
	pool IIa 400 ml	pool IIb 1,250 ml	
Extraction according to *Tanner and Gupta* (17)		(1) Extraction of free steroids (2) β-Glucuronidase enzyme hydrolysis	
Enzyme hydrolysis	Enzyme hydrolysis	(3) Solvolysis	Enzyme hydrolysis
Addition of radioactively labelled steroids as internal standards			
Extraction with ethyl acetate			
Separation of the phenolic steroids			
Celite column chromatography			
Multiple thin-layer chromatography			
Removal of aliquots to correct for procedural losses			
Gas-liquid chromatography			
Gas-liquid chromatography – mass spectrometry			

Table III. Thin-layer chromatography of steroids from pool I

P	Pa Pe	17OH-P	17OH-Pe
T-8	T-7	T-8	
T-12	T-4	T-13	
T-17		T-10	
T-21		T-16	
T-19		T-21	T-21

Processing and Results of Pool I

For each 100 ml of newborn urine, 50 g of ammonium sulfate were added. The mixture was then extracted with ether/isopropanol 3:1 and further processed as described (17). The residue was dissolved in 0.2 *M* acetate buffer, pH 5.2 (18) and incubated with β-glucuronidase/aryl sulfatase (Boehringer, Mannheim). At first, 1 million Fishman units β-glucuronidase and 500,000 Whitehead units aryl sulfatase were added and the incubation carried out at 37 °C for 48 h. A second, 24-hour incubation followed after addition of the same amounts of enzymes. The extraction was performed with ethyl acetate after the addition of the radioactively labelled internal standards. Then separation of the phenolic steroids was accomplished with 1 *N* sodium hydroxide. The neutral residue was chromatographed on a 40-g celite column with system C-1. This gradient elution chromatography yielded three fractions. The first contained P, the second contained Pa and Pe, and the third zone contained the radioactivity of 17OH-P and 17OH-Pe. These fractions were further purified by thin-layer chromatography as shown in table III. Identification and quantitation were carried out by GLC on XE-60, QF-1, OV-3, OV-7 and OV-17 columns. Except for P, TMSi derivatives were made: The total amount of P measured was 2.24 μg with a recovery of 37.2 %. Therefore, the concentration of P in pool I was calculated to be 2.1 μg/l (table VI). Verification of identity was obtained by GLC – mass spectrometry comparison (16). Measurable amounts of Pa, Pe, 17OH-P and 17OH-Pe were not found. Taking the recovery and sensitivity of the procedure into account, it was calculated that the concentration of Pa and Pe had to be below 0.3 μg/l, and for 17OH-P and 17OH-Pe less than 5 μg/l.

Processing and Results of Pool IIa

The newborn urine was adjusted with conc. HCl to pH 5.2 and buffered with 0.1 vol of 0.2 *M* acetate buffer (18). β-Glucuronidase/aryl sulfatase (Boehringer, Mannheim) was added to yield a concentration of 1,500 Fishman units β-glucuronidase and 750 Whitehead units aryl sulfatase per ml of urine. After

17OH-Pe

T-8
T-13
T-10
T-16
T-21

ım sulfate were added.
3:1 and further pro-
0.2 *M* acetate buffer,
sulfatase (Boehringer,
ronidase and 500,000
ıbation carried out at
after addition of the
ed with ethyl acetate
:andards. Then separa-
odium hydroxide. The
umn with system C-1.
ıctions. The first con-
d zone contained the
ere further purified by
ation and quantitation
and OV-17 columns.
unt of P measured was
ıtration of P in pool I
ntity was obtained by
e amounts of Pa, Pe,
/ and sensitivity of the
ntration of Pa and Pe
s than 5 μg/l.

pH 5.2 and buffered
e/aryl sulfatase (Boeh-
f 1,500 Fishman units
per ml of urine. After

Table IV. Thin-layer chromatography of steroids from pool IIb

P	Pa Pe	P-diol	17OH-P	17OH-Pe
T-18 T-19	T-23 T-7 T-4	T-21 T-12	T-9 T-16 T-21 T-12	T-19 T-17

48 h of incubation, the same amounts of enzymes were added and the mixture was further incubated for 24 h. Tritium-labelled P-diol was then added and extraction was done with ethyl acetate. After phenolic extraction, the neutral residue was chromatographed on a 40-g celite column using system C-1. The P-diol fraction was then submitted to thin-layer chromatography in systems T-9, T-19 and T-16. Identical retention times for the P-diol TMSi derivative were found on XE-60, QF-1, OV-3 and OV-7 columns. The concentration of P-diol was determined to be 625 μg/l (table VI). Identification by GLC – mass spectrometry has been described elsewhere (16).

Processing and Results of Pool IIb

The newborn urine was brought to pH 8, and the free steroids were extracted with ether. The extract was adjusted to pH 5.2 with HCl and 0.1 vol of 0.2 *M* acetate buffer (pH 5.2) added (18). During the first 48 h the mixture was incubated at 37 °C with 500,000 Fishman units of β-glucuronidase (Serva, Heidelberg), then the incubation continued for 24 h after the addition of the same amounts of enzyme. Following an ether extraction, the water phase was submitted to solvolysis (19). As radioactive internal standards tritium-labelled P, Pa, Pe, P-diol, 17OH-P and 17OH-Pe were added to the three residues and celite column chromatography with system C-1 carried out separately followed by thin-layer chromatography as shown in table IV.

Similar to pool I, Pa, Pe, 17OH-P and 17OH-Pe were not detectable. According to the recovery of the radioactive internal standards and the sensitivity of the method, the concentration of Pa and Pe had to be below 2 μg/l, and for 17OH-P and 17OH-Pe below 5 μg/l. This refers to the glucuronide, sulfate and free fraction as well. Also P was not measurable. A recovery of 10.5 % was obtained and since the pool size was smaller than before, this could explain the result. P-diol was measured in the free, glucuronide and sulfate fraction. Identity with reference steroid was found as TMSi derivative on XE-60, QF-1, OV-3, OV-7 and OV-17 columns. The quantitative results are listed in table VI. GLC –

mass spectrometry agreement was established (16). The GLC analysis of the P and Pa/Pe fractions obtained from pools I and IIb revealed several peaks with slightly different retention times compared with the reference steroids. GLC – mass spectrometric analysis demonstrated the presence of 16-DP, 16-DPa and 16-DPe (16). It is known that formation of such steroids can be acid-catalyzed (20). Therefore, pool III was collected and any strong acid or alkaline conditions were avoided during the isolation and identification procedures.

Processing and Results of Pool III

The first step of the purification procedure was by means of an Amberlite XAD-2 ion exchange column according to *Bradlow* (12). The glass column was 120 cm long and had an internal diameter of 15 cm. The conical outlet was regulated by a stopcock. Above a thick glass wool plug, 3,000 g Amberlite XAD-2 (Serva, Heidelberg) were suspended in distilled water and the surface stabilized with a layer of glass wool. The 2.6 liters of newborn urine were allowed to percolate slowly through this column. The column was washed with 10 liters of distilled water until the effluent was nearly colorless. Then the steroids were eluted with 12 liters of methanol. After evaporation of the methanol the residue was resuspended with 0.2 *M* acetate buffer pH 5.2 (18) and incubated for 48 h at 37 °C with 100,000 Fishman units β-glucuronidase and 50,000 Whitehead units aryl sulfatase. The same amounts of enzymes were again added and the incubation continued further for 24 h. Before extraction with ethyl acetate, measured amounts of radioactively labelled P, Pa, Pe, P-diol, 17OH-P, 17OH-Pe, 16OH-P and 16OH-Pe were added as internal standards. A

Table V. Thin-layer chromatography of steroids from pool III

P	Pa Pe	P-diol	17OH-P	17OH-Pe	16OH-P	16OH-Pe 16OH-D
T-18	T-23		T-8		T-1	T-1
T-23	T-18		T-16		T-5	T-5
T-12	T-4				T-2	T-2
	T-8	T-9	T-7		T-11	
	T-22	T-19	T-14		T-20	
	T-18	T-17	T-9		T-18	
	T-21	T-13	T-3		T-21	
	T-16	T-21	T-21		T-16	
		T-16	T-16			
			T-4	T-4		
			T-16	T-16		
			T-15	T-15		

GLC analysis of the P
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aporation of the meth-
uffer pH 5.2 (18) and
its β-glucuronidase and
of enzymes were again
Before extraction with
lled P, Pa, Pe, P-diol,
s internal standards. A

II

16OH-P	16OH-Pe 16OH-D
T-1	T-1
T-5	T-5
T-2	T-2
T-11	
T-20	
T-18	
T-21	
T-16	

50-g celite column with system C-1 was used for the first separation step. Five fractions were collected and further purified as indicated in table V.

Since unlabelled reference steroids were chromatographed simultaneously on the same plate each time, it is known that P and 16-DP migrated similarly throughout these extensive purifications. The same was found for Pa, Pe, 16-DPa and 16-DPe. Identity for all these steroids was obtained by GLC on QF-1, OV-3 and OV-7 columns. Except for P and 16-DP, TMSi derivatives were formed. The quantitative results are listed in table VI. GLC – mass spectrometric data are published elsewhere (16). Again in this pool Pa, Pe, 17OH-P and 17OH-Pe could not be measured. Using the same criteria as previously stated, the concentration of Pa and Pe had to be below 0.8 μg/l, and for 17OH-P and 17OH-Pe below 1.5 μg/l.

Table VI. Steroid concentration in urine of newborns

Steroid/pool	μg found	Recovery, %	μg/liter
P/I	2.2	37.2	2.1
P/III	1.9	15.9[1]	4.6
P-diol/IIa	250.0	38.7	625.0
P-diol/IIb glucuronide	490.0	65.0	605.0
P-diol/IIb sulfate	12.7	40.0	25.4
P-diol/IIb free	2.6	49.8	4.2
P-diol/III	388.0	20.5	729.0
16OH-P/III	675.0	36.4	713.0
16OH-Pe/III	705.0	1.9[2]	14,000.0
16OH-D/III	768.0	12.5	2,350.0
16-DP/I	136.0	37.2[3]	155.0
16-DP/IIb glucuronide	11.4	43.0[3]	21.2
16-DP/IIb sulfate	37.5	30.4[3]	97.5
16-DP/III	5.2	42.2[3]	5.3
16-DPe/I	876.0	80.6[3]	382.0
16-DPe/IIb glucuronide	1,260.0	73.0[3]	1,380.0
16-DPe/IIb sulfate	58.0	26.5[3]	172.0
16-DPe/III	179.0	39.0[3]	174.0
16-DPa/I	18.5	80.6[3]	8.3
16-DPa/IIb glucuronide	–		
16-DPa/IIb sulfate	79.0	26.5[3]	239.0
16-DPa/III	–		

– = Measurable amounts were not found.
[1] Losses on the Amberlite ion exchange column are included.
[2] Because of the huge quantity of steroid present, only a small fraction was purified.
[3] Recovery data are based upon the results of the corresponding nondehydrated steroids.

Discussion

The placenta is the main source of P within the feto-placental unit (21–23). P has been isolated and identified from amniotic fluid (1, 2). The concentration was found to be between 40 and 170 μg/l (1, 2, 24, 25). Recently, progestin levels measured by a competitive protein-binding technique were reported in amniotic fluid throughout pregnancy (26). We determined the P concentration in cord plasma and found an average concentration of 132 μg/100 ml (6, 7). Lower levels have been published previously (27–30), and an arteriovenous difference of cord plasma P concentrations has been demonstrated (28, 29, 31). We have now identified and quantitated P in newborn urine pools and determined a concentration between 2.1 and 4.6 μg/l (table VI). These levels are considerably lower than have been described for the first and second days of life by *Lauritzen and Schaper* (32). This same group also reported that on the third day, P could no longer be detected in most of the urine samples. Since the pools studied by us were collected from day 1 through 5 of life, a dilution effect has to be taken into account. However, part of the high concentration in the study by *Lauritzen and Schaper* (32) could have been caused by 16-DP as we shall demonstrate later. The lower concentration of P in newborn urine than in amniotic fluid is explained by the fact that the newborn is removed from the placenta, the main source of P biosynthesis. This finding also agrees with our concept that the amniotic fluid steroid pattern is mainly determined by fetal urinary steroid excretion. Since high concentrations of P are present in the fetal circulation, the excretion of this steroid will also be increased in fetal urine. Only trace amounts of P were found in meconium (33). This fact and our data on newborn urine allow us to postulate that P is not a major excretory product.

Pe has been found in the fetal circulation in large quantities (3, 5–7), and the concentration is higher in the umbilical artery than in the vein (5). This indicates fetal Pe production. Meconium and feces from infants throughout the first few weeks of life also contain relatively large amounts of Pe (33, 34). In spite of this, no Pe could be measured in newborn urine by us. The present investigation validates the findings in amniotic fluid in which we had been unable to detect Pe (1, 2).

A similar pattern has been established for D. Considerable quantities of this steroid are present in cord blood (3, 4, 6, 7, 30, 35–37). In amniotic fluid, however, only little was found (1, 2), and the same has also been confirmed for newborn urine (9).

Pa has been determined in newborn urine and measured in rather high quantities (38). We were unable to detect this steroid in three large pools of newborn urine which were collected during the first 5 days of life. In spite of extensive purifications (table V), the Pe/Pa-fraction revealed, after TMSi derivative formation and GLC, a number of compounds (fig. 1). Two of these have

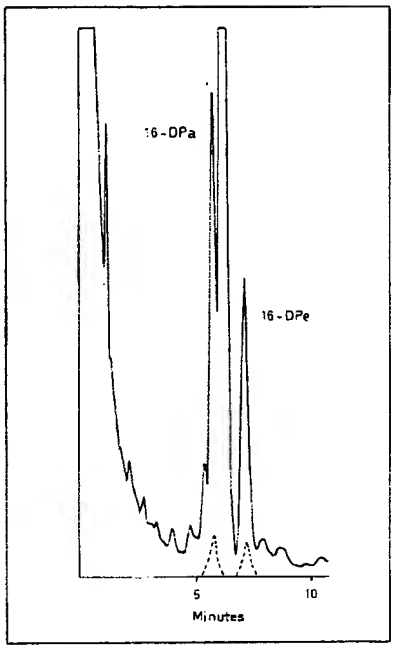

Fig. 1. Gas chromatogram of the Pe/Pa fraction of pool IIb sulfate on a column packed with Gas-Chrom Q coated with 3 % QF-1. – – – – – = Standard steroids.

been identified (16-DPe, 16-DPa). This fact could explain the high values for Pa obtained by others (38) using less specific means of separation and quantitation. Neither 3α- nor 3β-hydroxy-5β-pregnan-20-one had identical retention times with any of the other peaks. Therefore, from our studies we have to conclude that Pa can at most be present in small quantities in newborn urine. A similarity with Pe is obvious from our results.

Early studies on P-diol in newborn urine failed to detect this steroid even after considerable load with P (39, 40). Later this metabolite has been measured in newborn urine by several groups (38, 40, 41). Our studies with the pooled newborn urine reveal a P-diol concentration of 625–729 μg/l. It is noteworthy that fractional hydrolysis and combined enzyme hydrolysis (β-glucuronidase/aryl sulfatase) on the subdivided urine pool II yielded nearly identical results (table VI) and that the result obtained with pool III is also similar. The various amounts of enzymes used appear to have been sufficient since nearly equal amounts of P-diol with the different methodological approaches have been found. We determined that less than 1 % is present in the free fraction, about

3 % in the sulfate fraction, and 96 % were measured in the glucuronide fraction. For comparison, the glucuronide of P-diol seems to be the only conjugate present in the urine of pregnant women (42). In amniotic fluid, P-diol glucuronide appears to be the predominate conjugate (2). P-diol has also been measured in cord blood (6, 7, 29, 43). It exists there as sulfate and glucuronide in nearly equal concentrations (3), and in meconium mainly as sulfate (44). A similar pattern as we have found for the free and conjugated moieties of P-diol in newborn urine has been reported for estriol in fetal urine and amniotic fluid (45). This again confirms our findings that the steroid patterns in amniotic fluid and newborn urine are nearly equal and agree with recent data reported for corticosteroids (46). Therefore, the conclusion seems to be on firm grounds that toward the end of pregnancy the steroid content of amniotic fluid is mainly determined by fetal urinary steroid excretion. Since it was shown that there is a decrease of P-diol concentration in newborn urine for the first 5 days of life (38, 41), we compared our results with studies where urine was examined over the same period of time. This reveals a close agreement of the data from the literature and our study (38, 41).

Within the feto-placental unit, 17OH-P and 17OH-Pe are synthesized by the fetus. This has been shown by *in vitro* and *in vivo* experiments (47, 48). Also small amounts of 17OH-Pe and 17OH-P have been isolated from hydatidiform mole tissue *in vitro* (49), and 17OH-Pe has been shown to be synthesized by the normal placenta (21). Measurements of 17OH-P in cord venous and arterial plasma support these data (28). Similar to adult plasma (50), small amounts of these steroids are present in the fetal circulation (27, 28). This may explain the inability to detect 17OH-P and 17OH-Pe in newborn urine.

A very low concentration of 16OH-P has been described in cord blood (27); we have confirmed this (51). However, we did isolate relatively large amounts of 16OH-P from newborn urine (table VI). This also agrees with our own results on amniotic fluid in which a substantial quantity of this steroid was found (55). Again, the amounts of 16OH-P found in newborn urine correlate with the concept that amniotic fluid steroid content is determined by fetal urinary excretion and that the steroid patterns of amniotic fluid and newborn urine are similar. This is also borne out by our findings on 16OH-Pe and 16OH-D in this study. Both steroids were measured in large quantities (table VI). Previous studies have already shown this (8, 10, 52–54, 56). The same is true for amniotic fluid (1, 2). The concentration of 16OH-P in newborn urine is higher than that of 16OH-D (table VI). This agrees with results of others (52, 53, 56). In studies by *Shackleton et al.* (9) it was demonstrated that in the first 2–3 days of life, 16OH-D is higher in newborn urine. Later in life the reverse is true. Since our pools contained urine up to the 5th day of life, these data agree with our findings.

10 years ago, *Francis et al.* (58) isolated 16-DPe from meconium. It had been isolated before from equine urine (59). Recently, it was again described in

meconium and feces (34, 60, 74, 75) and isolated from human fetal bile (76). Trace amounts have been described in newborn urine (9) and it seems to be utilized as precursor in steroid biosynthesis (61).

16-DP has been described as a metabolite of 16OH-P *in vivo* in humans (63). It was also found in porcine ovarian tissue (64, 65) and in studies of the feto-placental unit (66, 67). The third Δ^{16}-C_{21}-steroid identified in our studies (16-DPa) has, to our knowledge, not been described before, but the isomers, 3β-hydroxy-5α-pregn-16-en-20-one and 3α-hydroxy-5β-pregn-16-en-20-one, have been isolated (59, 72). It seems well established that Δ^{16}-C_{19}-steroids are naturally occurring metabolites in animal and man (68–70, 73), but it is not certain if this is true for Δ^{16}-C_{21}-steroids. We have demonstrated that Δ^{16}-C_{21}-steroids are obtained by procedural manipulation. However, our studies also give evidence for the fact that at least 16-DP and 16-DPe do occur as natural metabolites in newborn urine (table VI). It has been clearly shown that 16-DPe is formed from 16OH-Pe by acid hydrolysis (71). This occurs with both the sulfate and free moieties. Since 16-hydroxylated steroids are major metabolic products of the fetus, enough substrate is present to form Δ^{16}-C_{21}-steroids by the methods used. We could demonstrate this by our study (table VI). But even under most careful handling (pool III), 16-DP and 16-DPe were isolated and quantitated. Therefore, we have to conclude that they are naturally occurring metabolites and that our results confirm previous findings (9, 58, 66, 67). Although recent data on biosynthesis of 16-DPe in fetal liver strengthen this point (57), 16-DPa was only found when strong acid conditions were used. Therefore this steroid might not be a naturally occurring metabolite.

We established the identity of these compounds by GLC on a number of different columns in part after TMSi derivative formation as well as by GLC – mass spectrometry (16). It is most likely that other isomers are present, but identification of the other GLC peaks (fig. 1) is not completed so far. Since microbial metabolism of Δ^{16}-C_{21}-steroids has been demonstrated (62), it remains to be evaluated whether intestinal microorganisms of the newborn are capable to form Δ^{16}-C_{21}-steroids.

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